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Evidence for dimer formation by an amphiphilic heptapeptide that mediates chloride and carboxyfluorescein release from liposomes

Robert Pajewski,[a] Riccardo Ferdani,[a] Jolanta Pajewska,[a] Natasha Djedovič,[a] Paul H. Schlesinger[b] and George W. Gokel*[a,c]

[a] *Department of Molecular Biology & Pharmacology, Washington University School of Medicine, 660 S. Euclid Ave., Campus Box 8103, St. Louis, MO 63110, USA; Fax: 14/362-9298 or 7058; Tel: 314/362-9297. E-mail: ggokel@molecool.wustl.edu*

[b] *Department of Cell Biology and Physiology, Washington University School of Medicine, 660 S. Euclid Ave., Campus Box 8103, St. Louis, MO 63110, USA*

[c] *Department of Chemistry, Washington University School of Medicine, 660 S. Euclid Ave., Campus Box 8103, St. Louis, MO 63110, USA*

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Heptapeptides having dioctadecyl, *N*-terminal hydrocarbon chains insert in phospholipid bilayer membranes and form pores through which at least chloride ions pass. Although amphiphilic, these compounds do not typically form vesicles themselves. They insert in the bilayers of phospholipid vesicles and mediate the release of carboxyfluorescein. Hill analysis indicates that at least two molecules of the amphiphile are involved in pore formation. In CD_2Cl_2, dimer formation is detected by NMR chemical shift changes. The anion release activity of individual anion transporters is increased by linking them covalently at the *C*-terminus or, even more, by linking them at the *N*-terminus. Evidence is presented that either linked molecule releases chloride from liposomes more effectively and rapidly than the individual transporter molecule at a comparable concentration.

Introduction

Many early designs for synthetic ion-conducting channels[1] focused on the construction of tubular or cylindrical structures that were conceptualized as "ion tunnels." Clever and sometimes elaborate synthetic strategies brought such designs to reality.[2] Once in hand and chemically characterized, however, the problem of demonstrating membrane insertion and transport efficacy was difficult and often remained unfulfilled. Other challenges include determining selectivity, channel insertion, functional dynamics and the mechanism(s) by which the synthetic channel compounds transport an ionic species. The latter is a particularly difficult enterprise that has occupied students of protein channels for decades.

We have developed two different families of channel-forming compounds. The molecules that we call "hydraphiles" use crown ethers as headgroups, entry portals, and as the intra-membrane ion relay and were designed to conduct alkali metal cations.[3] The latter is akin to the "water and ion-filled capsule" recently identified in the structure of the KcsA channel of *Streptomyces lividans*.[4] Over more than a decade, we have characterized the action of hydraphiles in planar bilayers, in liposomes, and most recently in live cells.[5] We have also found that the hydraphiles exhibit structure-dependent and selective toxicity to several different organisms.[6]

The second family of channels comprises membrane-anchored heptapeptides. The peptide sequence was designed to mimic the GxxP sequence that is conserved in the putative ion pathway of the ClC family of chloride-selective protein channels. An extensive effort has been undertaken in this synthetic channel family to characterize transport, selectivity, and mechanism. The essential elements of the channel design include an *N*-terminal hydrophobic anchor, a connector unit that mimics the phospholipid's midpolar regime, the heptapeptide sequence, and a *C*-terminal ester or amide. The first compound prepared is illustrative and has the following structure: $[CH_3(CH_2)_{17}]_2NCOCH_2OCH_2CO$-$(Gly)_3$-Pro-$(Gly)_3$-$OCH_2Ph$, **1**,[7] to which we have given the name *s*ynthetic *c*hloride *m*embrane *t*ransport *r*eceptor: SCMTR.

We have varied the structural elements of the compounds in this family in order to better understand the molecular function. We found that **1** was selective for chloride over potassium by at least 10-fold in phospholipid bilayers.[7] Compound **1** also exhibits voltage dependent gating.[7] When the anchor chains are systematically shortened, conductance increases because selectivity is diminished and both cations and anions are transported.[8] When proline in the heptapeptide sequence is replaced by leucine[9] or by pipecolic acid, a dramatic decrease in activity is apparent even though the change in the latter case from a five- to a six-membered ring seems to be a minor alteration.[10] When the *C*-terminal benzyl group is replaced by ethyl or isopropyl, activity is greatly diminished, but it is enhanced when benzyl is replaced by *n*-heptyl or *n*-decyl. Size specific dextran blocking experiments and Hill plots both suggested that **1** functioned as a dimer to form a pore of approximately 8 Å diameter.[10] We now report a study to further characterize dimer formation for the active heptapeptide. The results presented comprise a combination of physical studies and the design and preparation of two synthetic dimer mimics.

Results and discussion

Structural considerations

The three essential design elements of **1** are as follows. First, the twin hydrocarbon chains were incorporated to serve as lipid anchors that should insert into the phospholipid bilayer and align with the bilayer's lipid chains. Second, the diglycolic acid residue has three oxygen atoms and mimics the midpolar or glyceryl regime of natural phospholipids both in overall size and in polarity. The similarity of this group to the glyceryl ester region of phospholipids was expected to help position the monomers within the bilayer. Third, the heptapeptide incorporates the GxxP sequence described above and the central proline provides a bend in the heptapeptide chain. Computer calculations using Gaussian 98W and a detailed examination of molecular models both clearly showed the proline-imparted bend that we believe is critical to forming an opening in the bilayer.

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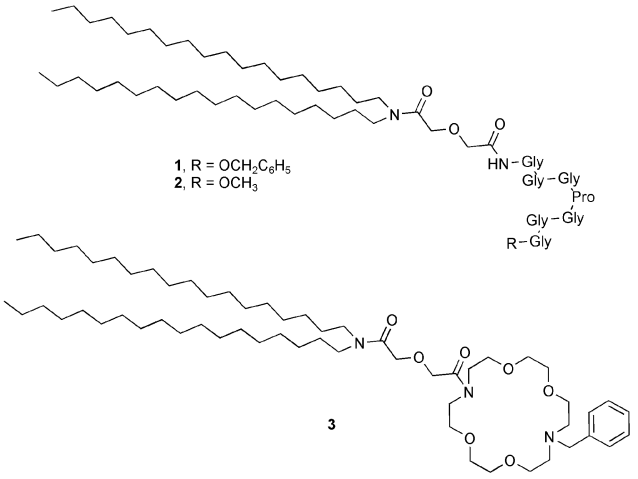
Overall, **1** is amphiphilic; the heptapeptide comprises the polar headgroup. We note, however, that we were unable to form stable vesicles from an aqueous suspension of **1**. However, by substituting a crown ether, which is known to be a headgroup in the amphiphilic sense,[11] for the heptapeptide, we formed compounds having the hydrocarbon and diglycolic acid spacer units of **1**. Specifically, we found that $[CH_3(CH_2)_{17}]_2NCOCH_2$-$OCH_2CO\langle N18N\rangle CH_2Ph$ ($\langle N18N\rangle$ is shorthand for diaza-18-crown-6),[12] **3**, formed vesicles of about 170 nm when suspended in water at 3 mM concentration and then sonicated.[13] These observations confirm the function of the hydrocarbon tails in **1**.



Because of the proline-induced bend, two heptapeptide chains could associate to form a diamond-shaped headgroup assembly. Obviously, this would be dynamic and would have a minimum size of 7–8 Å as judged by molecular models. The latter has been confirmed by dextran sizing. We note, however, that carboxyfluorescein (CF, ~10 Å) can pass through this pore, suggesting that it can enlarge slightly and still maintain integrity.[10] Fig. 1 shows the presumed organization of the two molecules of **1** in the phospholipid bilayer. The pore could be organized either by aligning the *N*-terminal and *C*-terminal ends or by organizing like termini in proximity. The two possibilities are illustrated schematically in Fig. 1.

Fig. 1 Schematic representation of the heptapeptides of **1** organized head (arrowhead) to tail or head to head (tail to tail).

Carboxyfluorescein release from liposomes mediated by 1

Carboxyfluorescein (CF) is an anionic dye at pH = 7 that has been used extensively as a fluorescent probe. Like chloride, it is an anion in these vesicle suspensions. Examination of molecular models suggests that CF is about 10 Å across while the hydrated radius of chloride is reported to be about 6.5 Å.[14] Although CF is clearly not Cl^-, CF has the advantages that it is anionic, it is readily detectable and quantitated, and it is a common probe for biological assay. It may be incorporated into the aqueous compartment of liposomes, in which its fluorescence is self-quenched. Replacement of the external aqueous phase removes free CF. When **1** inserts in the liposomal bilayer, CF is released. The appearance of CF external to the liposome may be monitored quantitatively by its fluorescence emission. We have previously reported the concentration dependent release of CF from unilamellar liposomes (182 ± 12 nm) prepared from 30% (w/w) 1,2-dioleoyl-*sn*-glycero-3-phosphate and 70% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine mediated by **1**.[10] The concentration range studied was 12.6–253 μM, or about a 20-fold variation. A typical series of curves for CF dequenching[8] is shown in Fig. 2.

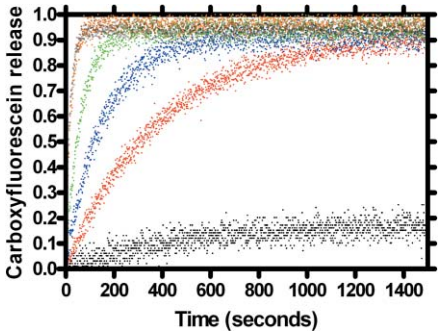


Fig. 2 Carboxyfluorescein release mediated by **1** at the following concentrations (bottom to top): 12.6, 25.3, 63.3, 127.0, 190.0, and 253.0 μM.

The fluorescent emission at $\lambda = 520$ nm (F_{520}) was monitored over more than an hour. The graph of Fig. 2 shows the release over only 1500 s. Total dye release is determined by vesicular lysis using the detergent Triton X-100 (1%), as described in the Experimental section. The data points shown are normalized to a final release value of 1.0 (*i.e.*, 100%) after lysis. Using the data so acquired, it is possible to obtain time constants for dye release. Experimental data were subjected to non-linear least squares analysis to determine the time constant of the exponential dequenching. Specifically, the data were fitted to the equation

$$F_{\lambda\,=520} = F_{\text{time}\,=0} + A \cdot (1 - e^{\text{time}/\tau}) + m \cdot \text{time}$$

as described previously.[15,16] The variables in this equation are: observed fluorescence (at $\lambda = 520$ nm, *i.e.*, $F_{\lambda\,=520}$), fluorescence at time zero ($F_{\text{time}\,=0}$), the size of the exponential component (A), the time constant for pore activation (τ), and the slope of the linear dequenching portion of the curve (m). Analysis of the kinetics relies upon the fact that the exponential portion of these curves represents those vesicles that empty from a single pore activation event. Under this constraint, pore activation is the rate-limiting step, which defines the value of tau.

Hill plot analysis of CF release data

The time constants obtained from the carboxyfluorescein release experiments were evaluated using Hill analysis to determine the number of SCMTR molecules required for pore activation. A Hill plot is a velocity equation normalized for the V_{max} that, when plotted in a log form, generates a slope characterizing the reaction's molecularity. In the present case, we interpret this slope to be the aggregation state or extent of monomer association for pore activation by **1** (*i.e.*, "*n*" in $\mathbf{1}_n$). Such an analysis can be meaningful when the concentrations vary by at least 10-fold. In the present case, data were obtained over a 20-fold concentration range. The logarithmic form of the equation[17] is:

$$\log_{10}[v/(V_{max} - v)] = n \cdot \log_{10}[S] - \log_{10}(K_{app})$$

In this equation, v = the velocity of anion release, V_{max} = the maximum velocity, n is the slope, [S] is the concentration of **1**, and K_{app} is the apparent association constant for pore formation. The data were fitted statistically to obtain the Hill plot. The graph shows a plot of $\log_{10}[v/(V_{max} - v)]$ *vs.* $\log_{10}$ [**1**] (see Fig. 3) over the concentration range 12.6–253.0 μM. This suggests that at least two molecules of SCMTR are involved in the pore activation.

Evidence for dimer formation in homogeneous solution

The evidence presented above for dimer or aggregate formation within the bilayer begs the question of whether or not **1** can associate in homogeneous solution. We showed in previous work that $R_2NCOCH_2OCH_2CO$-Gly-Gly-Gly-Pro-Gly-Gly-Gly-OR′

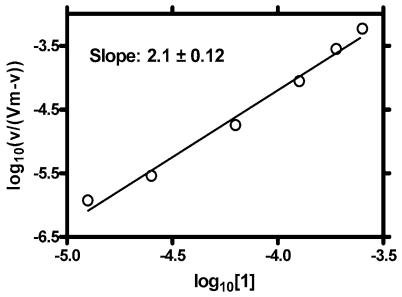


Fig. 3 Plot of CF release rate *vs.* decadic log of the concentration of **1**.

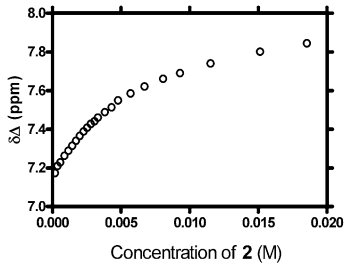


Fig. 5 Chemical shift dependence of the glycine proton (indicated glycine in –GGGP*G*GG–) on the concentration of **2** in CD_2Cl_2.

complexes ion pairs in $CHCl_3$ solutions.[18] Compound **1** was characterized by NMR when it was first prepared. ^{1}H-NMR signals for the *C*-terminal benzyl group and certain amide NH hydrogens overlap. We therefore prepared $[CH_3(CH_2)_{17}]_2$-$NCOCH_2OCH_2CO$-Gly-Gly-Gly-Pro-Gly-Gly-Gly-OCH_3, **2**, the ester group of which shows only a singlet at 3.72 ppm and permits the amide protons to be observed. Deuterated methylene chloride (CD_2Cl_2) was chosen in part because of its low dielectric constant ($\varepsilon \sim 9$) and also because **2** is very soluble in it. The residual proton signal at ~5.3 ppm does not obscure the NH signals. Experiments were conducted by diluting a 20 mM stock solution of **2** to final concentration of 0.2 mM. Significant shifts in the NH group ^{1}H-NMR signals upon dilution were consistent with dimer formation. ^{1}H-NMR spectra are shown for concentrations of 0.5 mM (lower trace) and 5.0 mM in Fig. 4.

The NMR shift data at various concentrations are shown in Fig. 5 and were fitted to the following equation:[19]

$$\delta_{obs} = \delta_{dimer} + \{(\delta_{monomer} - \delta_{dimer})[(-1 + (1 + 8K_A C)^{1/2}]/(4K_A C)\}$$

where C corresponds to the concentration of **2** and K_A, $\delta_{monomer}$, and δ_{dimer} are the calculated values. In other studies, the proton that is shifted was identified by deuteration of each amino acid in the heptapeptide (data not shown). In this case, we monitored the NH of the glycine on the *C*-terminal side of proline. The change in chemical shift as a function of concentration is shown in Fig. 5. When the data obtained were fitted to the equation shown, an association constant (K_A) of $59 \pm 2\ M^{-1}$ was obtained. We do not have data on the association of **1** in a phospholipid bilayer but this experiment comports with the formation of a dimer pore.

Considerations in the design of a covalent "dimer"

It seemed possible that the formation of a dimer in the phospholipid bilayer could be mimicked by covalent connection of two amphiphilic heptapeptides related to **1** or **2**. Forming a dimer by N → C terminal connections could be accomplished by preparing a tetradecapeptide of the sequence GGGPGGGGGGPGGG. The system would require to be anchored at the *N*-terminal end by a diglycolic acid unit attached to dioctadecylamine. How to anchor the *C*-terminus is problematic because the R_2-$NCOCH_2OCH_2CO$– unit would be present as an ester rather than as an amide and it would replace the typical *C*-terminal benzyl group. The effect of these alterations would mean, at a minimum, that instead of having two anchors and two esters, only one of each would be present. The influence on "head-group" structure of a dozen flexible glycine residues in a single chain was also an obvious issue. Of course, linking two molecules of **1** by any means presents some variant of this problem.

From the synthetic perspective, strategies for linking two SCMTR molecules could involve linking the *C*- or *N*-terminal ends. A bridge near the *N*-terminus (such as that in **4**) has the advantage of leaving the *C*-terminal benzyl groups intact to function as secondary anchors. It has the potential disadvantage that it holds the four hydrocarbon chains more closely together than they might be in separate monomers of **1**. Linking in this fashion could be accomplished by replacing diglycolic acid with iminodiacetic acid, $HOCOCH_2NHCH_2COOH$. The two nitrogen atoms could be bridged without compromising most of the structural features but two tertiary amines would be introduced near the hydrocarbon anchors. We believe that the hydrocarbon chains at the *N*-terminus embed themselves in the nonpolar regime of the bilayer. Protonation of these amines at physiological pH could increase the polarity in that portion of **4** compared to **1**. The pK_A of iminodiacetic acid's nitrogen atom is 9.1[20] so the nitrogen should be protonated to the extent of about 97% at pH = 7.4. Although longer N ↔ N linkers might have been chosen, the ready availability of ethylenediaminetetraacetic acid (EDTA) gave an excellent starting vehicle for the synthesis.

A two-carbon linker could also be used to connect the *C*-terminal ends of **1** to give **5**. This strategy leaves the anchor chains separated from each other so they may organize in an optimal fashion within the bilayer. Structure **5** has two obvious disadvantages, however. First, the *C*-terminal esters of **1** are converted to amides. Second, the short connector chain replaces two residues that are believed to function as secondary anchors (see discussion below). In the absence of experiment, it is unclear whether **4** or **5** would function more effectively in the bilayer,

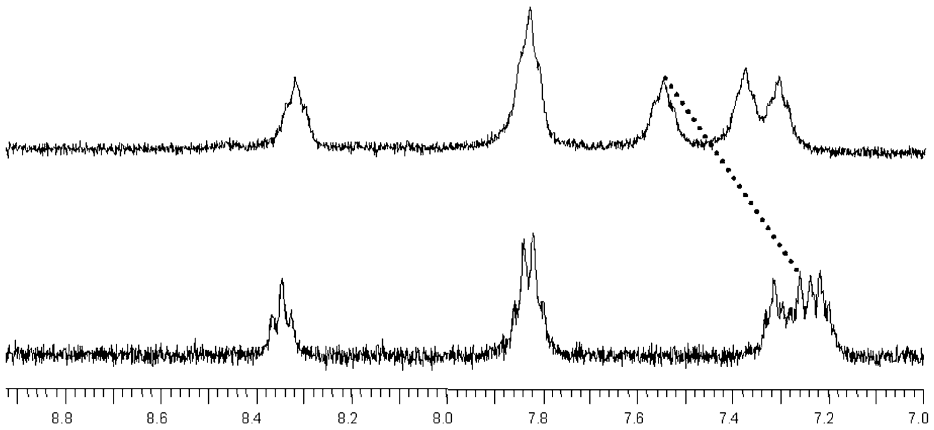
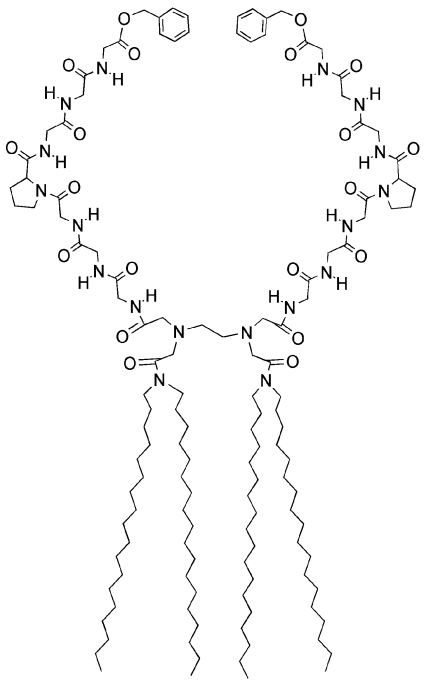
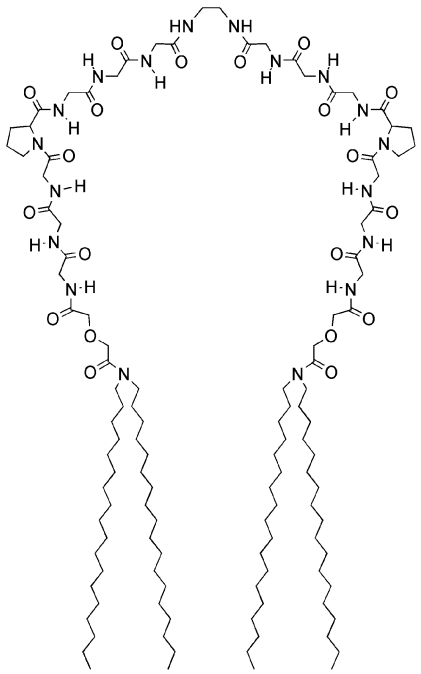


Fig. 4 ^{1}H-NMR spectra of **2** at 0.5 mM (lower panel) and 5.0 mM (upper panel) in CD_2Cl_2 solution.



4



5

but both should be better than an identical concentration of **1**. Tactically, the preparation of **5** is simpler than that of **4**, but both compounds have molecular weights over 2000 Daltons and present significant challenges.

Synthesis of compounds 4 and 5

Compound **4** was prepared as follows. The dianhydride of EDTA was heated under reflux in THF with dioctadecylamine to give the combined bridge and anchor units in 80% yield. The resulting diacid diamine was then coupled to H_2N-Gly-Gly-Gly-OCH_2Ph using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) and Et_3N (76%) and hydrogenolyzed (H_2, Pd/C, 95%) to remove the benzyl group and form the bis(acid). A final coupling between the diacid and the hydrochloride salt of Pro-Gly-Gly-Gly-OCH_2Ph gave **4** in 50% yield as a white solid, mp 126–128 °C. The sequence is illustrated in Scheme 1.

The preparation of dimer **5** was more straightforward. Compound **1** was debenzylated by treating with H_2 and Pd/C in 94% yield. The resulting acid was coupled in a 2 : 1 molar ratio with ethylenediamine to give **5** (76%) as a white solid, mp 205–207 °C. Details are given in the Experimental section for the preparations of both **4** and **5**.

Chloride release from liposomes mediated by 4 or 5

Fig. 6 plots fractional chloride release from phospholipid liposomes *vs.* time for compounds **1**, (*N*-linked) **4**, and (*C*-linked) **5**. In principle, when the two subunits derived from **1** are covalently held together, either at the *C*-terminus (**5**) or in the midpolar, diglycolic acid linker region (**4**), the two subunits are predisposed to form a dimer pore. Thus, faster pore formation kinetics are expected for both **4** and **5** compared to **1**. The formation of the pore is also favored thermodynamically. The covalent pre-assembly of the system lowers the entropy cost incurred when two molecules form one entity, such as a pore.

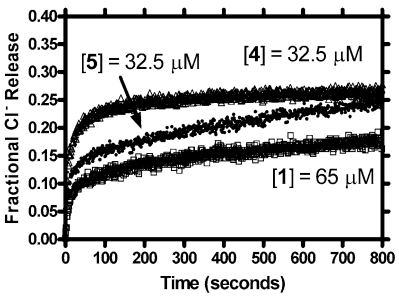
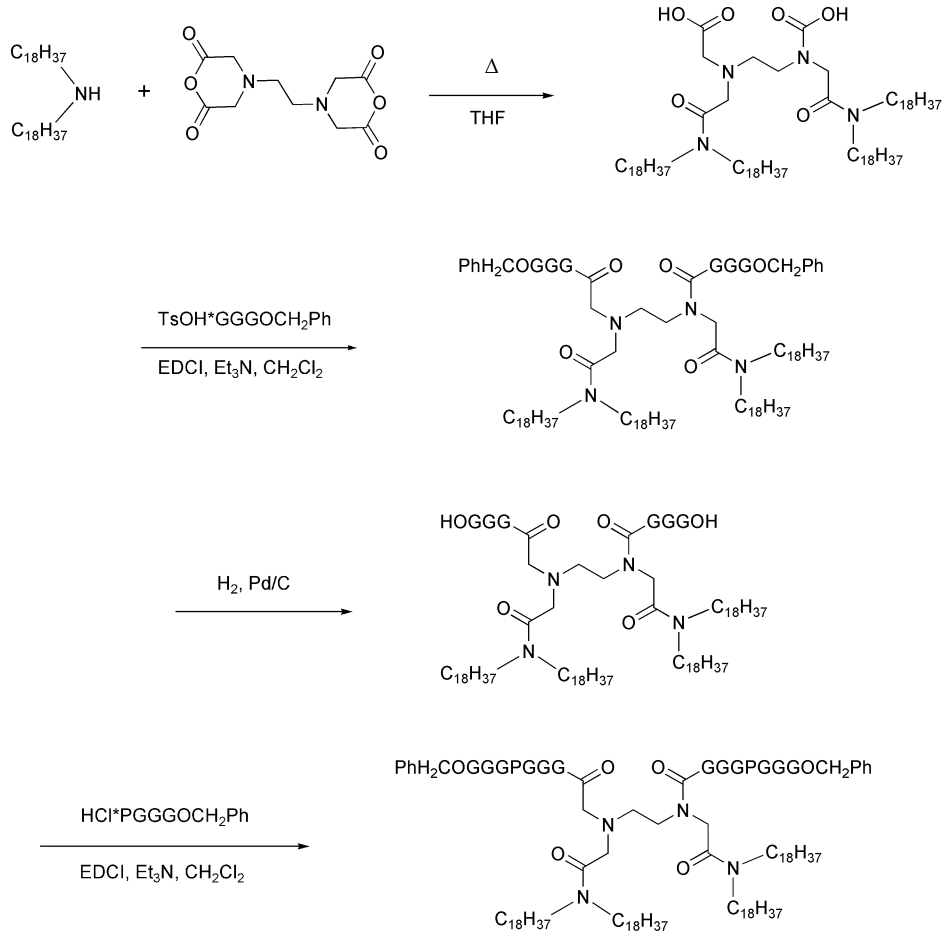


Fig. 6 Fractional chloride release from liposomes mediated by **4** and **5** at 32.5 μM concentrations and by **1** at 65 μM.

Both **4** and **5** release chloride from vesicles with faster kinetics and to a larger extent. *N*-Linked **4** is more active than is *C*-linked **5**. The difference may be due to the lack of a *C*-terminal anchor residue in **5**. We have recently studied the effect of changing the *C*-terminal residue in compounds related to **1**.[21] We assayed chloride ion transport in derivatives of **1** when the *C*-terminal ester (R in the structure above) was ethyl, isopropyl, heptyl, cyclohexylmethyl, or benzyl (**1**). The *C*-terminal heptyl ester was significantly more active than any of the other compounds, including the benzyl ester. Thus, the *C*-terminal ester residue appears to be a critical secondary anchor element in **1** and this is lacking in **5** but present in **4**. Molecular models of **4** and **5** suggest that the latter is more flexible than the former. Compound **4** possesses flexible chains but they are held in proximity by the linker unit and the *C*-terminal anchors further support the presumed pore conformation. The greater potential for flexibility is apparent in **5** compared to **4**. If **4** is more organized in the bilayer, it seems reasonable that pore formation kinetics should be faster.

When the hydrocarbon chains of **1, 2, 4**, or **5** insert in a phospholipid bilayer, they are unlikely to transcend the entire insulator regime of a membrane. We postulate that many molecules of amphiphile insert into the bilayer, but that individual monomers do not form pores. When two or more amphiphiles present in the leaflet come into proximity, pore formation is initiated. In the opposite leaflet of the bilayer, headgroup reorganization likely occurs to form a water-filled pore through the entire membrane. Since both **4** and **5** are already in proximity, pore formation should be faster for them than for **1**.

A comparison of chloride ion release by **1, 4**, and **5** was conducted in which the concentrations were adjusted to compensate for dimer pre-formation. Fig. 6 shows fractional chloride release for **1** at a concentration of 65 μM compared to **4** and **5** at concentrations of 32.5 μM. The initial release is clearly greatest for **4** but the chloride release curves are more similar than different. Certainly, chloride release is greater for either **4** or **5** than for **1** even though the latter is present at twice



Scheme 1 Preparation of **4**.

the concentration. This is not conclusive evidence for dimer formation *in situ* but comports with the other data presented in this study.

Conclusion

Compound **1** is a heptapeptide that is known to selectively conduct chloride ion through phospholipid bilayers. The heptapeptide chain is polar and the *N*-terminal hydrocarbon chains are hydrophobic. Such compounds insert in phospholipid bilayer membranes and apparently associate (aggregate) to form pores through which ions pass. By using fluorescent measurements to assay carboxyfluorescein release from vesicles, Hill analysis showed that pore formation involves at least two molecules of **1**. A solution ^{1}H-NMR study of **2**, the methyl ester compound related to **1**, suggested that the monomer formed a dimer in CD_2Cl_2. Two heptapeptide units were linked together at either their *N*- (**4**) or *C*-termini (**5**) to mimic the presumed dimer formation of **1** *in situ.* Compounds **4** and **5** release chloride ions from vesicles and both are more active than monomer **1**, even when **1** is present at twice the concentration of **4** or **5**. Taken together, the data confirm aggregate formation of molecules such as **1** and suggest a dimer mechanism for ion transport through the bilayer.

Experimental

General

^{1}H-NMR spectra were recorded at 300 MHz in $CDCl_3$ solvents and are reported in ppm (δ) downfield from internal $(CH_3)_3Si$. ^{13}C-NMR spectra were recorded at 75 MHz in $CDCl_3$ unless otherwise stated. Infrared spectra were calibrated against the 1601 cm^{-1} band of polystyrene. Melting points were determined on a Thomas Hoover apparatus in open capillaries and are uncorrected. Thin layer chromatographic (TLC) analyses were performed on aluminium oxide 60 F-254 neutral (Type E) with a 0.2 mm layer thickness or on silica gel 60 F-254 with a 0.2 mm layer thickness. Preparative chromatography columns were packed with activated aluminium oxide (MCB 80–325 mesh, chromatographic grade, AX 611) or with Kieselgel 60 (70–230 mesh). Chromatotron chromatography was performed on a Harrison Research Model 7924 Chromatotron with 2 mm thick circular plates prepared from Kieselgel 60 PF-254.

All reactions were conducted under dry N_2 unless otherwise stated. All reagents were the best (non-LC) grade commercially available and were distilled, recrystallized, or used without further purification, as appropriate. Molecular distillation temperatures refer to the oven temperature of a Kugelrohr apparatus. Combustion analyses were performed by Atlantic Microlab, Inc., Atlanta, GA, and are reported as percentages. Where water is factored into the analytical data, spectral evidence is noted for its presence.

Fluorescence studies with carboxyfluorescein

Liposomes were prepared by using the reverse phase procedure of Szoka and Papahadjopoulos.[22] A lipid mixture (10 mg) composed of 30% 1,2-dioleoyl-*sn*-glycero-3-phosphate and 70% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, AL) was dissolved in 0.5 mL of diethyl ether. To this was added 0.5 mL of 20 mM carboxyfluorescein in 100 mM KCl : 10 mM HEPES (pH = 7.0); the pH was adjusted to 7.0 with KOH. This mixture was sonicated (1200 W) for 3 × 20 seconds at 20 °C to produce a stable emulsion. The ether was removed at reduced pressure on a water aspirator (15 °C) in a round bottomed flask rotating at 40 rpm. The 0.5 mL suspension was supplemented

with an additional 0.5 mL of the 20 mM carboxyfluorescein solution. This mixture was passed five times through a 200 nm nucleopore filter. The extra-vesicular carboxyfluorescein was removed by passing the liposome–dye mixture over a 1 × 20 cm Sephacryl G-25 column (Sigma) in 100 mM KCl : 10 mM HEPES (pH = 7.0). The liposome peak was collected and analyzed by dynamic light scattering to give a diameter of 182 ± 12 nm. Phospholipid concentration in this fraction was determined to be 3.3 mg mL^{-1}.[23] Prior to use, carboxyfluorescein vesicles were diluted to 0.66 μg mL^{-1} in a reaction volume of 500 μL. The fluorescence (excitation 497 nm: emission 520 nm at 2 nm bandpass) was monitored at 25 °C. Compounds were added as a solution in isopropyl alcohol (5 mM) with mixing to the indicated concentrations. Data were digitized at 10 points per second and subsequently reduced to 1 point per second by decimation. Dequenching, F_{520}, was calculated as the fraction of total release upon addition of 1% Triton X100:

$$F_{520} = (F - F_0)/(F_{\text{Triton}} - F_0)$$

where F_0 and F_{Triton} are the zero time and Triton X-100 produced fluorescence. Dequenching data were fit using a nonlinear least squares method based upon the Levenberg–Marquardt algorithm, which generated χ^2 values indicating the goodness of fit for each group of averaged data sets. The number of individual trials generating the data set (degrees of freedom) was used to obtain the p value for the individual fits and kinetic constants. In all cases, the χ^2 values for individual fits were less than 0.05 and the resulting p values varied from 0.05 to 0.001 depending upon the number of trials. Data were fit to the equation shown in the text, as described previously.[14,15]

Studies of chloride release from liposomes

The lipids used in these studies, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA), were obtained as $CHCl_3$ solutions from Avanti Polar Lipids. A dry film of DOPC–DOPA (20 mg, 7 : 3 w/w) was dissolved in Et_2O (0.5 mL) and then 0.5 mL of internal buffer (600 mM KCl : 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH adjusted to 7.0) was added. Sonication (10 s) gave an opalescent dispersion. The solvent was evaporated *in vacuo* at 30 °C. The suspension was filtered (200 nm filter, 5 times) using a mini extruder and passed through a Sephadex G25 column, previously equilibrated with external buffer (400 mM K_2SO_4 : 10 mM HEPES, pH adjusted to 7.0). The vesicles obtained were consistently ~200 nm, as measured by a Coulter N4MD submicron particle analyzer. The final lipid concentration was determined by the colorimetric procedure of Stewart.[23]

Chloride release was assayed by using a chloride selective electrode (Accumet Chloride Combination Electrode) on the vesicles (0.31 mM) prepared as described above. The electrode was introduced in a 2 mL vesicles solution and allowed to equilibrate. The voltage at time zero was recorded and the system was allowed to stabilize for 5 min. Aliquots of the peptide (~9 mM in 2-propanol, ≤20 μL 2-PrOH) were added. Triton X-100 (100 μL, 2% solution) induced vesicular lysis. Data were collected using a DigiData 1322A series interface and Axoscope 9.0 software.

Compound **1** was prepared as previously described.[10]

Preparation of $(C_{18}H_{37})_2NCOCH_2OCH_2CONH$-Gly-Gly-Gly-Pro-Gly-Gly-Gly-OCH_3, 2

Debenzylation of 1 by hydrogenolysis. 18_2[DGA]-GGGP-GGG-OCH_2Ph, **1**, (0.75 g, 1.1 mmol) was dissolved in EtOH (abs., 30 mL) and 10% Pd/C (0.1 g) was added. The mixture was shaken under 60 psi pressure of H_2 for 3 h, heated to reflux, and then filtered through a celite pad. Solvent was evaporated under reduced pressure to afford 18_2[DGA]-GGGPGGG-OH as a white solid (0.65 g, 94%), mp 167 °C (dec.). ^{1}H-NMR: 0.84 (6H, t, $J = 6.9$ Hz, CH_3), 1.22 (60H, m, $CH_3(CH_2)_{15}CH_2CH_2N$), 1.50 (4H, bs, $CH_3(CH_2)_{15}CH_2CH_2N$), 1.80–2.20 (4H, m, Pro $NCH_2CH_2CH_2$), 3.06 (2H, t, $J = 7.5$ Hz, $CH_3(CH_2)_{16}CH_2N$), 3.26 (2H, t, $J = 7.5$ Hz, $CH_3(CH_2)_{16}CH_2N$), 3.00–4.40 (19H, Pro $NCH_2CH_2CH_2$, Gly CH_2, $COCH_2O$, Pro CH), 7.50 (1H, t, $J = 6.0$ Hz, NH), 7.69 (1H, t, $J = 6.0$ Hz, NH), 7.90–8.05 (2H, m, NH), 8.32 (1H, bs, NH). ^{13}C-NMR: 14.1, 22.7, 25.1, 26.9, 27.1, 27.6, 28.8, 29.3, 29.4, 29.6, 29.7, 31.9, 41.6, 41.9, 42.6, 42.8, 43.4, 46.4, 47.0, 61.2, 69.0, 70.9, 168.6, 168.9, 170.4, 170.8, 171.0, 171.3, 172.6, 173.7. IR ($CHCl_3$): cm^{-1} 3306, 3083, 2918, 2850, 1730, 1658, 1651, 1646, 1540, 1467, 1412, 1378, 1338, 1241, 1130, 1030, 909, 722.

Esterification to form 2. 18_2DGA-GGGPGGG-OH (0.30 g, 0.28 mmol) was suspended in CH_2Cl_2 (20 mL) and cooled to 0 °C. Diisopropylcarbodiimide (0.056 mL, 0.36 mmol), dimethylaminopyridine (DMAP, 0.02 g, 0.2 mmol) and MeOH (0.2 mL) were added and the mixture was stirred for 0.5 h then the ice bath was removed and the reaction stirred for 2 days at room temperature. The solution was evaporated and the residue purified by column chromatography (silica, $CHCl_3$–CH_3OH 95 : 5 → 85 : 15) to give the final product (0.27 g, 90%), as a white solid, mp 150–152 °C. ^{1}H-NMR: 0.88 (9H, t, $J = 6.9$ Hz, CH_3), 1.25 (60H, m, $CH_3(CH_2)_{15}CH_2CH_2N$), 1.48 (4H, m, $CH_3(CH_2)_{15}CH_2CH_2N$), 1.85–2.22 (4H, m, Pro $NCH_2CH_2CH_2$), 3.08 (2H, t, $J =$ 7.5Hz, $CH_3CH_2(CH_2)_{15}CH_2N$), 3.27 (2H, t, $J = 7.2$ Hz, $CH_3CH_2(CH_2)_{15}CH_2N$), 3.57 (1H, m, Pro $NCH_2CH_2CH_2$), 3.60 (1H, m, Pro $NCH_2CH_2CH_2$), 3.72 (3H, s, $COOCH_3$), 3.75–4.15 (14H, m, Gly CH_2, $COCH_2O$), 4.30 (2H, s, $COCH_2O$), 4.36 (1H, m, Pro CH), 7.36 (1H, bt, NH), 7.50 (1H, bt, NH), 7.71 (1H, bt, NH), 7.88 (2H, m, NH), 8.41 (2H, bt, NH). ^{13}C-NMR: 14.2, 22.8, 25.3, 27.0, 27.2, 27.8, 28.9, 29.1, 29.4, 29.5, 29.7, 29.7, 31.9, 41.4, 42.0, 42.8, 43.0, 43.6, 46.4, 47.0, 51.6, 61.3, 61.6, 69.4, 71.3, 168.6, 168.9, 170.2, 170.5, 170.9, 171.1, 173.7. IR ($CHCl_3$): 3301, 2919, 2851, 1745, 1654, 1546, 1467, 1411, 1377, 1335, 1243, 1130, 1030, 908, 733 cm^{-1}. Anal. Calcd. for $C_{58}H_{106}N_8O_{11}\cdot H_2O$: C 62.79, H 9.81, N 10.10. Found: C 62.79, H 9.81, N 10.10%.

Compound **3** was previously reported.[13]

Preparation of $(C_{18}H_{37})_2NCOCH_2N(CH_2CONH$-Gly-Gly-Gly-Pro-Gly-Gly-Gly-$OCH_2Ph)CH_2CH_2N(CH_2CONH$-Gly-Gly-Gly-Pro-Gly-Gly-Gly-$OCH_2Ph)CH_2CON(C_{18}H_{37})_2$, 4

Preparation of $(C_{18}H_{37})_2NCOCH_2N(CH_2COOH)CH_2CH_2N$-$(CH_2COOH)CH_2CON(C_{18}H_{37})_2$, tetraoctadecyl-EDTA, intermediate 4a. A solution of dioctadecylamine (3.0 g, 5.75 mmol), ethylenediaminetetraacetic dianhydride (0.73 g, 2.85 mmol) and NEt_3 (3.0 mL) was refluxed in THF (100 mL) for 48 h. The solvent was then evaporated and the crude product dissolved in $CHCl_3$ and washed with a diluted solution of HCl. The solvent was removed and the residue recrystallized from ethyl ether to give **4a** product as a white solid (2.96 g, 80%), mp 66–68 °C. ^{1}H-NMR ($CDCl_3$–CD_3OD ≈ 2 : 1): δ 0.83 (12H, t, $J = 6.9$ Hz, CH_3), 1.21 (120H, m, $CH_3(CH_2)_{15}CH_2CH_2N$), 1.51 (8H, m, $CH_3(CH_2)_{15}CH_2CH_2N$), 3.15 (4H, t, $J = 8.1$ Hz, $CH_3(CH_2)_{15}CH_2CH_2N$), 3.26 (4H, t, $J = 8.1$ Hz, $CH_3(CH_2)_{15}CH_2CH_2N$), 3.74 (4H, s, NCH_2CH_2N), 4.21 (4H, s, $NC(O)CH_2N$), 4.41 (4H, s, $HOOCCH_2N$). ^{13}C-NMR ($CDCl_3$–CD_3OD ≈ 2 : 1): δ 13.8, 22.5, 25.7, 26.7, 26.9, 27.3, 28.4, 28.9, 29.2, 29.4, 29.5, 29.6, 31.8, 46.6, 47.3, 47.5, 51.5, 55.1, 55.8, 164.6, 168.0.

4b. $(C_{18}H_{37})_2NCOCH_2N(CH_2CONH$-Gly-Gly-Gly-$OCH_2$-$Ph)CH_2CH_2N(CH_2CONH$-Gly-Gly-Gly-$OCH_2Ph)CH_2CON$-$(C_{18}H_{37})_2$. To a solution of intermediate **4a** (0.80 g, 0.61 mmol) in CH_2Cl_2 (60 mL) cooled to 0 °C with an ice bath, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDCI, 0.26 g, 1.35 mmol), 1-hydroxybenzotriazole (HOBt, 0.19 g, 1.41 mmol), GGG-OBz tosylate (0.56 g, 1.24 mmol) and Et_3N (1.5 mL) were added and the mixture stirred for 1 h. The ice bath was then removed and the reaction continued

for 4 days. The reaction was quenched and washed with a saturated solution of citric acid (20 mL), a saturated solution of $NaHCO_3$ (20 mL) and water (20 mL), then dried over $MgSO_4$, evaporated and the residue purified by column chromatography (silica, $CHCl_3$–CH_3OH 95 : 5 → 9 : 1) to give the pure final product (0.86 g, 76.5%). ^{1}H-NMR: 0.88 (12H, t, J = 6.3 Hz, CH_3), 1.17 (120H, m, $CH_3(CH_2)_{15}CH_2CH_2N$), 1.45 (8H, m, $CH_3(CH_2)_{15}CH_2CH_2N$), 2.75 (4H, s, NCH_2CH_2N), 3.08 (4H, t, J = 8.1 Hz, $CH_3(CH_2)_{15}CH_2CH_2N$), 3.20 (8H, bs, $CH_3(CH_2)_{15}CH_2CH_2N$, $NC(O)CH_2N$), 3.47 (4H, s, $NC(O)CH_2N$), 3.89 (4H, d, J = 5.1 Hz, Gly CH_2), 3.93 (4H, d, J = 4.2 Hz, Gly CH_2), 3.99 (4H, d, J = 5.1 Hz, Gly CH_2), 5.10 (4H, s, $PhCH_2O$), 7.31 (10H, m, Ar-H), 7.49 (2H, bt, Gly NH), 7.86 (2H, bt, Gly NH), 8.72 (2H, bt, Gly NH). ^{13}C-NMR: 14.2, 22.8, 27.1, 27.3, 27.9, 29.1, 29.5, 29.6, 29.7, 29.80, 29.85, 32.1, 41.3, 43.1, 43.5, 46.7, 47.5, 54.8, 57.3, 59.6, 67.0, 128.3, 128.5, 128.7, 135.6, 169.3, 169.9, 170.0, 170.4, 173.3.

Intermediate 4c. Compound **4b** (0.46 g, 0.25 mmol) was dissolved in absolute ethanol (100 mL), 10% Pd/C (0.25 g) was added and this mixture was shaken under 70 psi hydrogen pressure for 18 h in a Parr apparatus. The reaction mixture was heated to reflux and filtrated through a celite layer, then the solvent was evaporated under reduced pressure to leave a white solid (0.39 g, 95.4%). ^{1}H-NMR and ^{13}C-NMR in $CDCl_3$ solution were very broad but there was no signal remaining that corresponded to the benzyl residue.

$(C_{18}H_{37})_2NCOCH_2N(CH_2CONH$-Gly-Gly-Gly-Pro-Gly-Gly-Gly-$OCH_2Ph)CH_2CH_2N(CH_2CONH$-Gly-Gly-Gly-Pro-Gly-Gly-Gly-$OCH_2Ph)CH_2CON(C_{18}H_{37})_2$, 4. Compound **4c** (0.71 g, 0.43 mmol) was suspended in CH_2Cl_2 (80 mL) and cooled to 0 °C. 1-(3-Dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (0.20 g, 1.04 mmol), 1-hydroxybenzotriazole (0.14 g, 1.04 mmol), PGGG-OBz·HCl (0.36 g, 0.87 mmol) and Et_3N (1.0 mL) were added and the mixture was stirred for 0.5 h then the ice bath was removed and the reaction stirred for 2 days at room temperature. The solution was washed with a saturated solution of citric acid (50 mL), a saturated solution of $NaHCO_3$ (50 mL) and water (50 mL), then dried over $MgSO_4$, evaporated and the residue purified by column chromatography (silica, $CHCl_3$–CH_3OH 95 : 5 → 8 : 2 → 8 : 2 + 5% NEt_3) to give the final product which was recrystallized from methanol to give a white solid (0.51 g, 50%), mp 126–128 °C. ^{1}H-NMR ($CDCl_3$–CD_3OD 9 : 1; NB, almost all of the peaks are quite broad): 0.84 (12H, t, J = 6.9 Hz, CH_3), 1.22 (120H, m, $CH_3(CH_2)_{15}CH_2CH_2N$), 1.45 (8H, m, $CH_3(CH_2)_{15}CH_2CH_2N$), 1.80–2.20 (8H, m, Pro $NCH_2CH_2CH_2$), 2.67 (6H, s, H_2O), 2.80 (4H, s, NCH_2CH_2N), 3.09 (4H, bt, $CH_3CH_2(CH_2)_{14}CH_2CH_2N$), 3.19 (4H, bt, $CH_3CH_2(CH_2)_{14}CH_2CH_2N$), 3.40 (solvent peak covering Pro $NCH_2CH_2CH_2$ and $NCOCH_2N$), 3.80–4.10 (24H, Gly CH_2), 4.31 (2H, bs, Pro CH), 5.10 (4H, s, $PhCH_2O$), 7.30 (10H, s, Ar–H), 7.71 (4H, m, Gly NH), 7.90 (2H, m, Gly NH), 8.01 (2H, m, Gly NH), 8.31 (2H, m, Gly NH), 8.66 (2H, m, Gly NH). ^{13}C-NMR: (as above, peaks are broad): 14.3, 22.9, 25.2, 27.1, 27.4, 29.1, 29.5, 29.7, 29.8, 29.9, 32.1, 41.4, 42.8 (very broad), 46.0, 46.6, 47.0, 47.5, 58.8 (broad), 61.5 (broad), 67.2, 128.4, 128.5, 128.7, 135.5, 169.4, 170.4, 170.8, 171.4, 173.9 (all the CO peaks are broad). Elemental analysis calcd. for $C_{130}H_{224}O_{20}N_{18}\cdot 3H_2O$: C, 64.70; H, 9.61; N, 10.45%. Found: C, 64.68; H, 9.75; N, 10.05%.

$[(C_{18}H_{37})_2NCOCH_2OCH_2CONH$-Gly-Gly-Gly-Pro-Gly-Gly-Gly-$NHCH_2]_2$, 5

Debenzylated **1** (see above, 0.21 g, 0.20 mmol) was suspended in CH_2Cl_2 (10 mL). 1-(3-Dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (0.04 g, 0.21 mmol) was added, followed by HOBt (0.03 g. 0.21 mmol) and the reaction was stirred for 0.5 h. Then 1,2-diaminoethane (6.6 μL, 0.09 mmol) in CH_2Cl_2 (50 mL) containing *N*-methylmorpholine (0.02 mL) was added and the reaction mixture was stirred for 48 h at room temperature. The solvent was evaporated and the residue was crystallized from MeOH to give **5** as a white solid (0.16 g, 76%), mp 205–207 °C. ^{1}H-NMR: 0.87 (12H, t, J = 6.9 Hz, $-CH_2CH_3$), 1.25 (120 H, pseudo-s, $CH_3(CH_2)_{15}CH_2CH_2N$), 1.51 (8H, bs, $CH_3(CH_2)_{15}CH_2CH_2N$), 1.80–2.25 (8H, m, Pro $NCH_2CH_2CH_2$), 3.07 (4H, t, J = 7.5 Hz, $CH_3(CH_2)_{16}CH_2N$), 3.26 (8H, overlapping signals due to $CH_3(CH_2)_{16}CH_2N$ and $CONHCH_2CH_2NHCO$), 3.40–3.45 (2H, m, Pro $NCH_2CH_2CH_2$), 3.50–3.55 (2H, m, Pro $NCH_2CH_2CH_2$), 3.85–4.05 (24H, m, Gly NCH_2), 4.10 (2H, s, $COCH_2O$), 4.29 (2H, s, $COCH_2O$), 4.35 (2H, bs, Pro NCH), 7.34 (2H, bs, Gly $CONH$), 7.81 (2H, br, Gly $CONH$), 7.91 (2H, br, Gly $CONH$), 7.97 (2H, bs, Gly $CONH$), 8.04 (2H, bs, Gly $CONH$), 8.36 (1H, bs, Gly $CONH$). ^{13}C-NMR: 14.1, 22.3, 24.9, 26.8, 26.9, 27.5, 28.6, 29.2, 29.5, 29.6, 31.8, 41.1, 41.8, 42.5, 42.6, 42.8, 43.1, 44.2, 46.1, 46.7, 61.1, 69.1, 70.9, 168.5, 169.0, 170.4, 170.5, 170.6, 170.9, 171.3, 173.6. IR (KBr): 3305, 3081, 2924, 2853, 1656, 1544, 1466, 1377, 1340, 1245, 1129, 1028, 720.54 cm^{-1}. Anal. Calcd for $C_{116}H_{212}N_{18}O_{20}\cdot H_2O$: C, 63.74; H, 9.82; N, 11.48%. Found: C, 63.36; H, 9.81; N, 11.39%.

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